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14. ABSTRACT

This first year of this award has been one of intense infrastructure building. This included recruitment and training of two excellent research associates, two PhD postdoctoral fellows, and one part-time student. I spent several months working closely with Dr. Donna Ferrandino on a human subjects protocol which was approved in August by both the Stanford IRB (protocol ID 4408) and DoD HSRRB (A-13776.2). This enabled us to work closely with our surgery and pathology colleagues to develop an efficient system of identifying, recruiting, and consenting subjects, and to obtain samples from the operating room to pathology and eventually to my laboratory. We tested multiple protocols to maximize recovery of immune cells from tumor and lymph node specimens. We also optimized methods for analysis of fresh and archive samples by flow cytometry, immunohistology, immunoflourescence, function assays, and DNA microarray analysis. The attached annual report is a summary of our progress in relation to my proposed SOW.

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Breast cancer, immunology, immunotherapy, systems biology

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Annual Progress Report 3/1/06-2/28/07
DoD Era of Hope Scholar Award
Immunology, Systems Biology, and Immunotherapy of Breast Cancer
Peter P. Lee, M.D.
Stanford University

INTRODUCTION
Breast cancer patients with similar tumor characteristics may have vastly different clinical courses, response to therapy, and outcome. Several lines of evidence now suggest that the host immune response may play a significant role in modulating disease progression in cancer. A complex interplay exists between the host immune response and tumor cells as a critical determinant in clinical outcome. These factors remain poorly understood. By comprehensively studying the dynamics between breast cancer and the immune response using an integrative systems approach, we hope to uncover opportunities for vastly different immunotherapy approaches than what are available today. We seek to move beyond the current paradigm of eliciting immune responses against defined antigens via vaccination, as this strategy alone does not appear to be effective in a number of clinical trials for melanoma. Rather, we seek strategies that specifically modulate tumor-immune cell interactions and block cancer-induced immune dysfunction on a systemic and local level (at tumor sites). In this project, we will use a number of novel immunological approaches to look for evidence of immune cell dysfunction within the tumor or tumor-draining lymph nodes (TDLNs) from breast cancer patients. This will include archived samples from patients with at least five year survival data, and fresh samples from newly diagnosed patients. We will use DNA microarrays to analyze the gene expression patterns of purified tumor and immune cells, focusing on gene networks and cross-talk between tumor and immune cells. We will generate high-resolution images of tumor and TDLN sections and develop image analysis algorithms to assess the spatial arrangement and grouping of tumor and immune cells with respect to each other that may have biological significance. Using statistics and mathematical tools, we will integrate the complex data generated from all of these studies and correlate them with clinical parameters. Lastly, our observations will be combined into a mathematical model that will enable us to perform *in silico* experiments to quickly test novel therapeutic strategies for breast cancer. This work may lead to novel diagnostic tools to help predict clinical outcome and guide therapy in breast cancer patients. We also hope to find new insights into the mechanisms of immune evasion by breast cancer cells and ultimately new treatment strategies for breast cancer directed specifically at altering the biology of TDLNs.

BODY
This first year of this award has been one of intense infrastructure building. This included recruitment and training of two excellent research associates, two PhD postdoctoral fellows, and one part-time student. I spent several months working closely with Dr. Donna Ferrandino on a human subjects protocol which was approved in August by both the Stanford IRB (protocol ID 4408) and DoD HSRRB (A-13776.2). This enabled us to work closely with our surgery and pathology colleagues to develop an efficient system of identifying, recruiting, and consenting subjects, and to obtain samples from the operating room to pathology and eventually to my laboratory. We tested multiple protocols to maximize recovery of immune cells from tumor and lymph node specimens. We also optimized methods for analysis of fresh

and archive samples by flow cytometry, immunohistology, immunoflourescence, function assays, and DNA microarray analysis. Below is a summary of our progress in relation to my proposed SOW.

Experiment Strategy
To fully understand tumor-immune cell interactions in breast cancer, our strategy is to compare the immune cells (and tumor cells) within three distinct compartments: the tumor, tumor-draining lymph nodes (TDLNs), and blood. We approach this at both the molecular and cellular levels. At the molecular level, gene expression profiling of immune cells and tumor cells within the tumor site and TDLNs will be carried out. At the cellular level, immunologic functions of immune cells will be studied and compared across these three compartments.

1. Immunological Analyses

Originally proposed in the SOW:
A. Analysis of archived samples of tumor and TDLN from breast cancer patients with at least 5 years of clinical follow-up data. Tumor and immune cell markers will be identified via immunohistochemical (IHC) staining and in-situ hybridization (ISH). Images will be acquired in high resolution using an automated imaging system (BLISS), and data will be acquired using automated software. Over 50 immune and tumor markers will be assessed. To facilitate these complex studies, we will also explore the use of tissue microarrays (TMA). This would enable us to analyze sections from 100-400 samples on each slide. We will first perform a pilot study to ensure that the TMA method is compatible for our studies and would not be negatively impacted by the architectural heterogeneity within TDLN. (months 0-60)
B. Analysis of live cells from fresh tumor, TDLN, blood, and possibly bone marrow from newly diagnosed or relapsed breast cancer patients undergoing surgery or treatment. Assays include flow cytometry (up to 12 colors), peptide-MHC tetramer analysis, sorting, functional responses (e.g. cytotoxicity, cytokine release, anergy, apoptosis, proliferation), and others. (months 6-60)
C. Generation of T cell lines and tumor cell lines from fresh tumor and TDLN samples for further detailed analyses. (months 6-60)
D. If the above studies demonstrate immune cell dysfunction within tumor or TDLN, but by themselves do not reveal any definitive mechanisms, then we will undertake in vivo studies with mouse models of de novo breast cancer to address the early events in immune dysfunction. (months 24-60)

Sample Acquisition
A total of 32 breast cancer patients have been enrolled into this study since September 8, 2006. All subjects were newly diagnosed without a history of any immune disorder prior to breast cancer diagnosis and had their surgical treatments at Stanford University Medical Center. Written informed consent has been obtained from all participants according to Stanford IRB, DoD HSRRB, and HIPAA regulations. Patients' heparinized peripheral blood samples, breast tumor tissue, tumor draining lymph node (TDLN: non-sentinel lymph node and/or sentinel lymph node aspirates) have been collected for the purpose of this study (Table

1). The clinical data for each participant is displayed in Tables 2a-c. Following surgical management, blood will be collected from these patients during a follow-up period of 5 years. The duration of disease-free survival (DFS) (between initial diagnosis and first recurrence) or relapse will be recorded as well.

Table 1. Number of Samples Collected and Range of Recovered Cells (n=32)

	# Samples Collected	Range of Recovered Cells
Blood	19	$12x10^6$ - $60x10^6$
SLN Aspirate	7	$0.3x10^6$ - $41x10^6$
Non-SLN	7	$10.1x10^6$ - $100x10^6$
Tumor	19	$0.2x10^6$ - $55x10^6$

Table 2a. Patient Characteristics

Age					Stage							
Range	Average	Median	<50	≥50	Stage 0	Stage 1	Stage 2a	Stage 2b	Stage 3a	Stage 3b	Stage 3c	Stage 4
32-81	53.8	53	12	19	2	6	3	0	3	1	7	4

Table 2b. Primary Tumor Characteristics

Histological Tumor Grade					Tumor Grade			ER Status		PR Status		Her-2/neu Status		Angiolymphatic Invasion	
Tis	T1	T2	T3	T4	Grade 1	Grade 2	Grade 3	Positive	Negative	Positive	Negative	Not expressed	Over expressed	None	Present
2	9	5	5	3	1	11	10	17	14	16	15	12	9	5	8

Table 2c. Lymph Node Characteristics

Nodal Stage				Tumor involved Lymph Nodes	
N0	N1	N2	N3	0	≥1
9	8	1	4	10	11

Sample Processing

The following procedures were used to optimize our sample processing. There are still some minor modifications needed before we can finalize the protocols. The range of cells recovered for each sample is summarized in Table 1. Because we are in the process of finalizing these protocols, the cells collected thus far are cryopreserved for further usage.

Breast Tissue dissociation and optimization

The dissociation condition was tested using various enzymes (Collagenase III/IV, DNase I, Hyaluronidase) at different concentration over 1 hr or 2 hrs to acquire more cells without jeopardizing their integrity. Weights of the tissues and the total number of recovered cells are

summarized in Table 3. Cell surface markers were stained and analyzed by flow cytometry. We found what we believe to be damaged cell surface markers during flow cytometry staining, possibly due to tissue digestion. To correct such damage, cells are allowed to rest overnight after digestion to fully recover cell surface marker expression, which has been confirmed by flow cytometry. The cell populations in breast tumor tissue include fibroblasts, epithelial cells, immune cells and less than 5% of endothelial cells, myoepithelial and myofibroblasts cells.

Table 3. Weights of Tumor Tissue and Number of Recovered Cells

Weight (g)	cells recovered ($\times 10^6$)	cells per gram ($\times 10^6$)
1	7.4	7.4
0.5	0.2	0.4
4(L); 1.5 (R)*	9 (L); 0.6(R)*	2.25(L);0.4(R)*
0.9	7.3	8.1
0.5	5.9	11.8
0.5	1.4	2.8
0.6	4.8	8
1	3	3
1.9	15	7.9
2	50	15
0.87	21	24.14
1.38 (R); 2.13 (L)*	57(R); 30(L)*	41(R);14(L)*
0.55	5.7	10

* Patient underwent bilateral mastectomy

Primary breast tumor cell line

We have attempted to establish short term cultures of breast epithelial cells *in vitro*. This allows propagation of sufficient quantities of cells with defined phenotype suitable for subsequent cell and molecular biology studies. In our case, cells are only maintained in culture for less than three months, which provides less opportunity to undergo the transformations that are seen in long-term culture of immortalized cell lines (Burdall *et al*, 2003).

Isolation of immune cells and tumor cells

First, we tried to isolate immune cells and tumor cells by negative selection using MACS beads. Although we were able to achieve a purity of 90%, we lost 50% of the cells due to processing. We are in the process of developing a multiple color panel for flow cytometric sorting. Ideally, cells would be sorted using the FACSAria to isolate the three major cell populations in breasts tumor tissue: immune cells ($CD45^+CD326^-CD140\beta^-$), epithelial/tumor cells ($CD326^+CD45^-CD140\beta^-$), and fibroblasts ($CD140\beta^+CD45^-CD326^-$). The same sorting panel will be utilized to isolate immune cells and epithelial/tumor cells in TDLNs.

Peripheral blood immune cell isolation

A different strategy will be employed to isolate immune cells from peripheral blood. Immune cells are usually separated by Ficoll-Hypaque density gradient centrifugation. However,

Ficoll-Hypaque is not only toxic and mutagenic, but also removes most of the granulocytes from peripheral blood. Because we would like to include all immune cell types, we are currently testing red blood cell (RBC) lysis buffer to isolate immune cells.

Flow cytometry-based Functional Assays of Lymphocytes

To assess the basic immunologic functions of human lymphocytes, an 11-color, 13 parameter flow cytometry-based functional assay was developed to measure proliferative responses and Th1/Th2 cytokine production of lymphocytes. Since the majority of immune cells in the tumor site, TDLN and PBL are T cells (50-70%) and B cells (5-30%), a functional assay was developed to study these specific cells.

a. CFSE-based proliferation assay
   The intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) is employed to monitor proliferation of lymphocytes. Cell division results in sequential halving of fluorescence, and up to 8 divisions can be monitored before the fluorescence is decreased to the background fluorescence of unstained cells. The relationship between cell division and cell function is readily measured at the time of analysis by using a cell function marker (cell surface or intracellular protein) labeled with an alternate fluorochrome.

b. Intracellular cytokine staining
   Th1 cytokines include IL-2, IFNγ, and TNFβ, which activate cytotoxic T cells and macrophages to stimulate cellular immunity and inflammation. Th2 cytokines include IL-4, IL-5, IL-6, and IL-10, which stimulate antibody production by B cells. In this study, IL-2 and IFNγ are used to represent Th1 cytokine, IL4 and IL10 for Th2 cytokine.

   The combination of proliferation assays and cytokine productions will allow us to identify the proportion of cells that are able to proliferate and the cytokines produced (Th1 vs Th2) following stimuli challenges. Furthermore, by blocking certain types of cytokine production using antibodies, this protocol can be applied to study the sequence of cytokine production from proliferating cells and therefore the interplay between CD4 T cells, CD8 T cells and B cells.

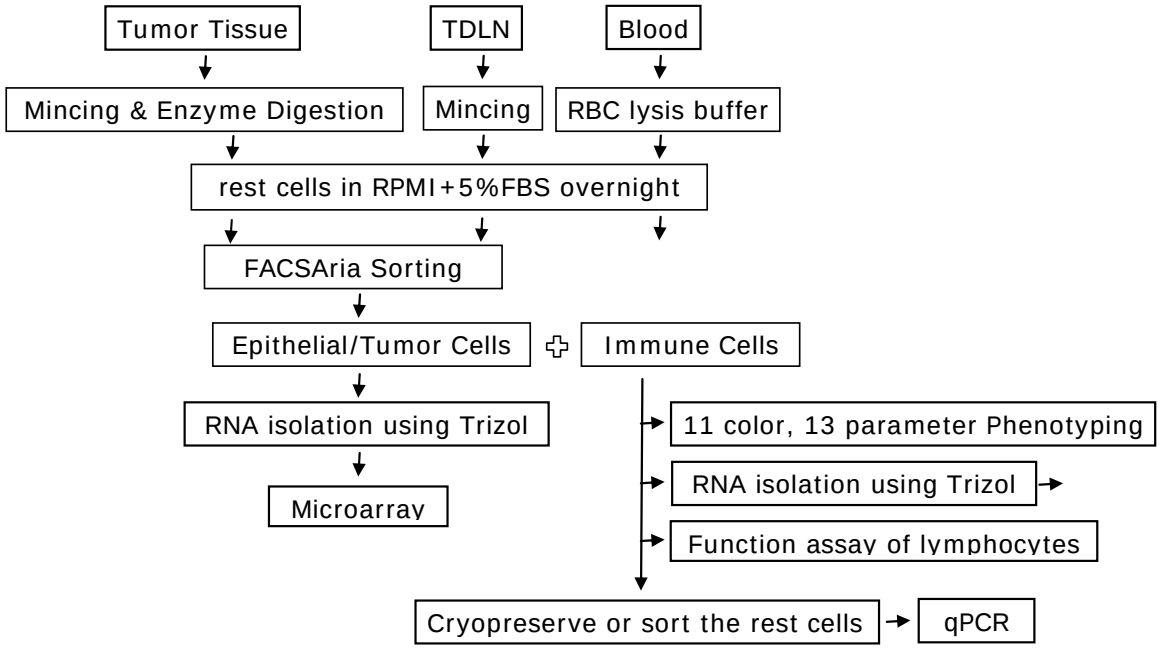
c. Optimization
   - A variety of stimuli (anti-CD28 beads, anti-CD3 beads, phytohemagglutinin, phorbol esters/Ionomycin, anti-CD40L, anti-IgM beads, lipopolysaccharide) were tested individually or in combination to achieve a resonable signal for both proliferation and cytokine secretion.
   - Proliferation and cytokine production are being monitored over time to determine the best stimulation period for proliferation and the best re-stimulation period for cytokine detection.
   - Protein transport inhibitors (Brefeldin A and monensin) for intracellular cytokine staining were compared since the characteristics of each compound is different in terms of cell viability, cell surface marker expression, and efficiency of inhibition for different types of cytokines.

- A new class of viability dye, the amine reactive viability dyes (ViD) is used and optimized as a dead cell exclusion marker. It has been shown that the fluorescence of cells stained with these dyes correlates with traditional dead cell discriminating markers, even after fixation and permeabilization (Perfetto *et al*, 2006).
- The marker fluorochromes were compared and carefully selected to achieve the best signal/noise level. Generally, brightly emitting fluorochromes (APC, PE) are reserved for cytokines not abundantly expressed such as IL4 and IL10. Tandem dyes are compared and titrated for each lot and from different companies since the fluorescence of tandem dyes varies significantly and tends to degrade quickly over time. The 11 color, 13 parameter flow cytometry panel is shown in Table 4.

Table 4. 11 color flow cytometry panel for functional studies

Antibody	Fluorochrome	
	CFSE	Proliferation
CD3	PE Cy7	T cell
CD8	PE-Alexa Fluor 700	Cytotoxic T cell
CD4	Pacific Orange	T helper cell
CD19	PE-Texas Red	B cell
CD69	PerCP	Activation marker
IL2	Alexa Fluor700	Th1 Cytokine
IFNr	APC-Alexa Fluor750	Th1 Cytokine
IL10	PE	Th2 Cytokine
IL4	APC	Th2 Cytokine
	ViVID	Dead cell discriminator

Outline of Sample Processing and Experimental Design is illustrated in the following flowchart.



Personnel: Lee, Johnson, Dirbas, Schwartz, Post-doc 1, research assistant 1, research assistant 2.

2. Microarray analysis of immune and tumor cells independently

Originally proposed in SOW:
A. Microarray analysis of gene expression of purified tumor and immune cells, isolated from fresh tumor or TDLN samples, and peripheral blood mononuclear cells (PBMC) from breast cancer patients. (months 6-60)
B. Detailed analyses of gene expression data focusing on gene networks and cross-talk between tumor and immune cells. (months 12-60)

a. A variety of microarray platforms are now available for whole genome gene expression profiling. However, the design of probes and arrays for each platform is quite different. Therefore, systematic biases arising from platform-specific parameters, such as measurement precision, accuracy, specificity and sensitivity or differences in protocol performance, should be weighted during the experimental design. Our strategy is to apply multiple microarray platforms to the same RNA samples. This will provide increased confidence when a gene expression change is discovered by multiple platforms. Equally important, this will avoid losing biologically significant information due to platform-specific performance variations. Such a strategy has recently been employed by several groups (Grigoriadis *et al*, 2006; Sorlie *et al* 2006). Two microarray platforms will be used for this study, Affymetrix Human Genome U133 (HG-U133) Set and Agilent Whole Genome Oligo Microarray (5989-0654EN). The Affymetrix HG-U133 set is the most widely used platform with gene expression information of 10,958 samples submitted to Gene Expression Omnibus (GEO). Ideally, these data in the public domain can be utilized to provide additional information complimentary to our findings. However, some technical difficulties have been encountered when we attempted to compare microarray data across groups/labs and platforms. Protocols of data normalization are being developed to resolve this issue.

b. The minimal and optimal number of immune/tumor cells for different microarray platforms were determined and summarized in Table 5. Ideally this will generate at least 100ng of total RNA, which will be enough for two arrays using Agilent Whole Genome Oligo Microarray or one array for Affymetrix Human Genome U133 A or B chip.

Table 5. Optimized No of Cells for Microarray Analysis

	Minimal No.	Optimal No.
Immune cells	4×10^4	2×10^5
Epithelial/Tumor cells	1×10^4	3.5×10^4

c. Protocols developed for these two platforms are described as follows:

Agilent Whole Genome Oligo Microarray:
Total RNA will be amplified in two consecutive rounds using Amino Allyl MessageAmp^{TM} II aRNA Amplification Kit (Ambio, Inc), followed by the Cy3/Cy5

labeling (Amersham Biosciences Corp., NJ) and hybridization according to the Agilent technical manual.

Reference RNA for Agilent microarray: Buffy coats will be obtained from 20 age-matched healthy female donors from the Stanford Blood Center. Immune cells will be purified as described above and total RNA will be isolated using the Trizol method. 100 μg total RNA from each sample will be pooled to create the immune cell reference RNA. Normal breast tissue will be collected from 10 breast reduction subjects and processed as described above. Epithelial cells will be isolated and total RNA purified using Trizol. 1 μg total RNA from each epithelial sample will be pooled to create the epithelial cell reference RNA.

Affymetrix Human Genome U133A or B chip:
Total RNA will be used in the first reverse transcription of two consecutive rounds of linear amplification with the protocol described by Baugh (Baugh *et al*, 2001), followed by fragmentation and hybridization according to the Affymetrix technical manual.

d. 11-color, 13-parameter flow cytometry phenotyping
Gene expression profiling of immune cells in the tumor site, TDLN and PBL is used as a first-pass in this study. Although the majority of immune cells from these compartments are T cells (50-70%), there are other types of immune cells (B cells, NK cells, Monocytes/Macrophages etc.), and the distribution of immune cell types in these compartments are slightly different. It is therefore important to know the distribution of immune cell types in these compartments. This will be very useful for interpreting the gene expression data.

A multiple color flow-cytometry phenotyping panel was therefore developed to analyze the distribution of immune cell types. For microarray analysis, 0.5 million cells of each sample will be phenotyped by this 11-color, 13-parameter flow cytometry panel. The cells/subsets of interest and surface markers used for phenotyping are shown in Table 6. The phenotyping result of PMBCs from a healthy donor is illustrated in Figure 1 as representative data.

Table 6. Surface marker for cells/subsets

Cells/Subsets of Interest	Surace marker
Cytotoxic T cells	CD3+CD8+CD4-
T helper cells	CD3+CD4+CD8-
Regulatory T cells	CD3+CD8-CD4+CD25highCD127low
Suppressor T cells	CD3+CD4-CD8+CD28-
B cell	CD19+CD3-
NK cell	CD3-CD19-CD56dimCD16high
Monocytes/Macrophages	CD14+CD3-CD19-
Dendritic cells	Lineage-DR+

e. Pilot study

Since we seek to compare gene expression profiles of cells across three anatomic compartments, which require different sample processing procedures, it is important to rule out influences from different sample processing. Currently, we are conducting a pilot study using a healthy donor PBMCs to find out the influences of cryopreservation, Ficoll-Hypaque density gradient centrifugation, RBC lysis buffer, enzyme digestion, coupled with overnight resting. The immune cells will be processed accordingly and stained to check for changes of cell surface marker expressions. In addition, a quantitative real time PCR (qPCR) will be used to check for gene expression changes of house keeping genes.

f. Real Time Quantitative PCR (qPCR)
The altered gene expressions of immune cells observed in the microarray experiments will be validated by real time quantitative PCR (qPCR).

As mentioned above, there are various types of immune cells (T cells, B cells, NK cells, monocytes/macrophages etc.) and the distributions of these cell types at different anatomic compartments are slightly different. Furthermore, each immune cell type may have a unique gene expression signature. Therefore, we will take one step further to validate the gene expression changes in each type of immune cells by sorting various cell types using the multiple flow cytometry panel mentioned above. As we can foresee, there will be only a limited number of cells available for qPCR. Single cell qPCR will be developed to solve this issue.

An equally important element in the success of this work is rigorous data analysis. Microarray analysis has led to generation of massive amounts of data. To derive biologically meaningful knowledge from such data, it is important to utilize state-of-the-art bioinformatics tools to access gene networks and biological correlations. This is itself a complex field in statistics. It would be impossible, let alone inefficient, for a post-doc with laboratory training to master the necessary bioinformatics tools and analysis. We are very fortunate to have a leader in this field, Professor Susan Holmes, as a close collaborator on this project. However, the data being generated is so vast that it would be impossible for her to perform all the necessary data transformation and analysis. This project would greatly benefit from a full-time post-doc (a third post-doc) with rigorous training in bioinformatics. Such a person will also be instrument in the integration of the disparate types of data being generated from the various aspects of this project into a cohesive set that can then be stringently correlated with clinical outcome data to uncover biological and immunological patterns that produce favorable clinical responses in breast cancer patients.

Personnel: Lee, Holmes, Johnson, Dirbas, post-doc 2, research assistant 1, research assistant 2. **A third post-doc would greatly enhance the success of this project.**

3. Epigenetic dysregulation

Originally proposed in SOW:
Assess alterations in epigenetic control of gene expression in immune cells (due to direct effects of tumor cells or to the general cancer state) isolated from fresh tumor or TDLN samples, and peripheral blood mononuclear cells (PBMC) from breast cancer

patients. This will be done using proprietary technologies from Orion Genomics – Methylscope and Methylscreen.

As stated in the SOW, pilot studies using these technologies will be conducted around year 2. If found promising, these studies will continue for the remainder of the award. However, our experience so far suggest that we may not have sufficient numbers of immune cells recovered from patient specimens to fully support these higher risk analyses. This stems from the current trends in the surgical management of breast cancer patients of removing fewer lymph nodes and patients with smaller tumors being detected. Both of these trends lead to smaller and fewer samples from breast cancer patients being available for research purposes, necessitating the need for us to optimize all of our assays and focusing on the highest yield experiments.

4. Analyzing the geometric relationships and interactions between cancer and immune cells in tumors and TDLN

Originally proposed in SOW:
A. Generate high-resolution images of tumor and TDLN sections. (months 0-60)
B. Develop algorithms to identify cells/cell types and assign coordinates. (months 0-60)
C. Develop algorithms to assess the spatial arrangement and grouping of tumor and immune cells with respect to each other that may have biological significance. This will be done in collaboration with a Stanford mathematics professor, Dr. Doron Levy, using advanced image analysis and computational geometry techniques. (months 0-60)

We have optimized 2- and 3-color immunohistochemical (IHC) staining combinations to concurrently visualize tumor cells and various immune cells within tumor and TDLN sections. A key accomplishment of the first year is the development of custom software to identify each cell type, its location, and enumerate the total numbers of tumor and immune cells within each section (figure 2). This custom software also enables us to quantify average distances between tumor cells and each immune cell type, and local densities of tumor and immune cells (figure 3). A key to the success of this portion of the project is access to a high resolution imaging system. While we have access to such a system (BLISS, Baccus Labs) through a collaboration, we only have use of 2-3 hours per day, which is sufficient to image just one slide per day. This is now a major bottleneck, which can be best addressed through the acquisition of an imaging system dedicated to this project.

Personnel: Lee, Levy, Schwartz, student, research assistant 1.

5. Synthesizing a useful model of breast cancer through mathematical and computational modeling

Originally proposed in SOW:
To integrate our experimental data and observations into a mathematical model to address the dynamics of cancer cells and the immune response in the tumor and lymph

node. This will ultimately enable us to perform *in silico* experiments to quickly test novel therapeutic strategies for breast cancer.

As stated in the SOW, these studies will commence around year 2 and will continue throughout the duration of this award.

Personnel: Lee, Levy, **a third post-doc would greatly enhance the success of this project.**

Outline of the project plan for the next 12 month

- Finalize the optimal sample processing procedures.
- Finalize the flow cytometry-based functional assays of lymphocytes.
- Optimize single-cell qPCR.
- Complete microarray analysis of an initial 20 patient sample set. Each set includes tumor cells, tumor infiltrating immune cells, immune cells from TDLN, and immune cells from blood.
- Complete functional assays of lymphocytes for sample acquired in the next 12 month. Ideally this should be performed using patients sample sets to compare the lymphocyte function across three anatomic compartments. If there is not enough tumor infiltrating immune cells, we will focus on immune cells from TDLN and blood.
- Develop functional studies for regulatory T cells.

Personnel

1. Peter P. Lee, MD – project PI (50% effort on EHSA)
2. Erich Schwartz, MD, PhD – Stanford Pathology (no salary requested on EHSA)
3. Denise Johnson, MD and Fred Dirbas, MD – Stanford Surgical Oncology (no salary requested on EHSA)
4. Susan Holmes, PhD – Stanford Statistics (1 month per year, as 33% of 3-month summer period)
5. Doron Levy, PhD – Stanford Mathematics (Year 1: 1 month per year, as 33% of 3-month summer period; Years 2-5: 2 months as 66% of summer period)
6. HongXiang Yu, PhD - post-doc 1, 100% effort on EHSA – immunological and histology studies
7. Rebecca Critchley, PhD - post-doc 2, 100% effort on EHSA – microarray and epigenetic studies, data analysis
8. TBN graduate student 1 (Immunology program), 100% effort on project but funded by fellowship - immunological and histology studies
9. TBN graduate student 2 (Stanford Mathematics), 100% effort on project but funded by fellowship - modeling and data integration
10. Diana Simons - research assistant 1, 100% effort on EHSA – to aid in immunological, histology, and microarray studies
11. Edina Levic - research assistant 2, 100% effort on EHSA – to aid in patient enrollment/consent, sample acquisition and processing

KEY RESEARCH ACCOMPLISHMENTS:

- Recruited an excellent team of 2 PhD post-docs, 2 research assistants, and 1 student.
- Human subjects protocol approved by Stanford IRB and DoD HSRRB.
- Developed an efficient system of identifying, recruiting, and consenting subjects, and to obtain samples from the operating room to pathology and eventually to my laboratory.
- Tested multiple protocols to maximize recovery of immune cells and tumor cells from tumor and lymph node specimens.
- Optimized methods for analysis of fresh and archive samples by flow cytometry, function assays, and DNA microarray analysis to study immune and tumor cells within tumor and TDLN specimens.
- Enrolled a total of total of 32 breast cancer patients into this study since September 8, 2006 (less than 6 months).
- Optimized 2- and 3-color immunohistochemical (IHC) staining panels for analysis of archived tumor and TDLN specimens.
- Developed custom image analysis software to identify each cell, cell type, location, and relate cell populations by distances and geometric patterns.

REPORTABLE OUTCOMES: On-going from efforts from this first year.

CONCLUSION:

As already mentioned, this first year has been one of intense infrastructure building. We have made substantial progress in developing a mechanism to efficiently recruit patients into this study, procuring their samples, and analyzing these samples via a powerful set of assays that we have adapted and optimized. One limitation that we encountered is the relatively small amounts of tissue available from most patients. This stems from the growing trend of early diagnosis and tissue preservation in the clinical management of breast cancer patients. Most of the small tumors and TDLNs are rightly used for clinical diagnosis, leaving very little for research. As a result, we are constantly trying to minimize the numbers of cells we need to generate useful data, and have to make decisions to pursue only the most promising assays with many samples. Nonetheless, we are beginning to uncover dramatic changes in the immune cell populations, which will provide important insights into how breast cancer alters the host immune system. We look forward in the coming year to generate substantial data from the infrastructure and assays that we now have in place.

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APPENDICES: None at this time.

SUPPORTING DATA: Tables are integrated into the text above. Three figures are presented in the following pages.

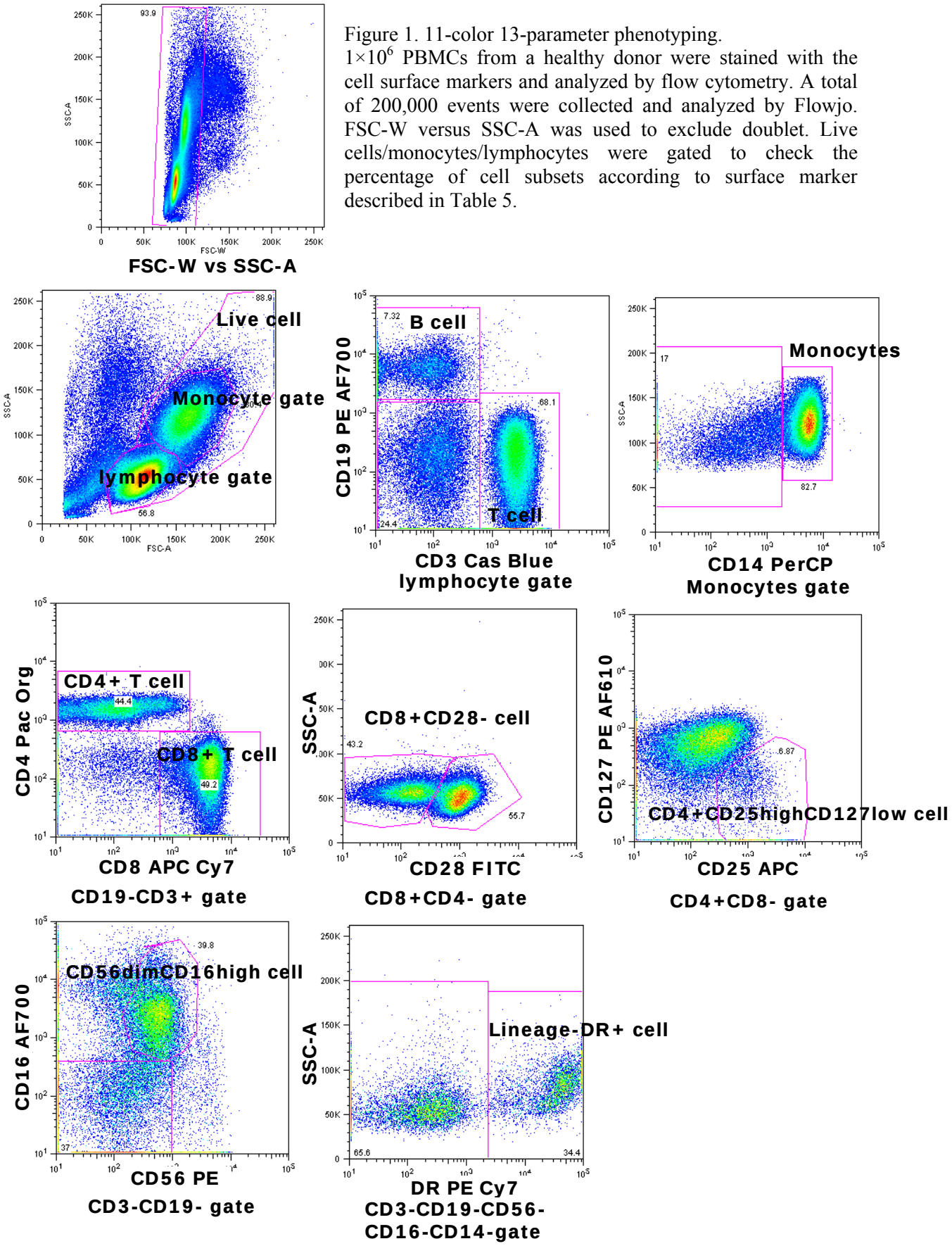

Figure 1. 11-color 13-parameter phenotyping.
1×10^6 PBMCs from a healthy donor were stained with the cell surface markers and analyzed by flow cytometry. A total of 200,000 events were collected and analyzed by Flowjo. FSC-W versus SSC-A was used to exclude doublet. Live cells/monocytes/lymphocytes were gated to check the percentage of cell subsets according to surface marker described in Table 5.

Figure 2. Examples of object identification within complex images by custom image analysis software. The left column shows images of oranges on trees, and the right column shows the microscopic images of tumor cells within a lymph node. The top row is the original image, the middle row shows identified oranges (left) or tumor cells (right) superimposed on the original image, the bottom row is the original image marked with the centroid locations of each identified object.

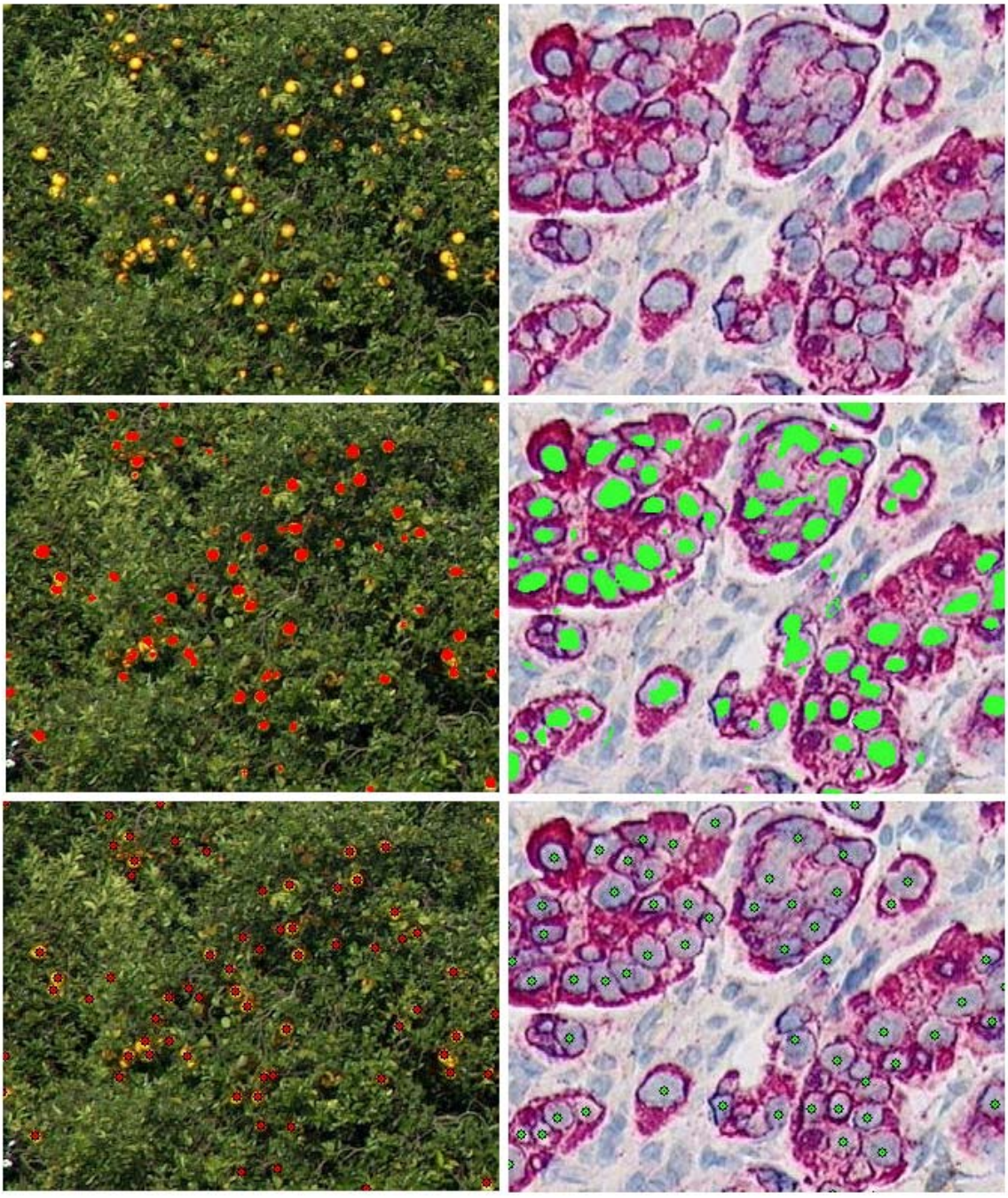

Figure 3. An example of the command-line data analysis panel (screen shot from software output). The histogram displayed within the panel is the result of a function that finds the distances from all T cells to their closest tumor neighbors. The binary image on the right is the cancer membrane for the entire slide - useful for clustering or other geometrical analyses. The open PDF document on the lower right is the auto-generated report which includes a thumbnail view of the entire image set, counts and Type I error rates for all phenotypes, as well as a transcript of the analyses performed.